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13. ABSTRACT ***(Maximum 200 Words)***

It is difficult to obtain good quality cDNA from RNA obtained from microdissected cells from pathological sections, particularly from paraffin sections. We are studying ways of isolating extremely small quantities of mRNA and/or 1st strand cDNA with magnetic beads. We are developing techniques for increasing the yield of 1st strand and 2nd strand synthesis reactions. In addition, we are studying 2 methods of amplification: the polymerase chain reaction and amplified RNA methods. Most of our work to date has been carried out with total RNA isolated by traditional methods. In the coming year, we will apply our findings to RNA or 1st strand cDNA obtained from pathological sections.

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INTRODUCTION: We proposed to explore new ways to synthesize cDNA libraries from microdissected single cells based on the technique of amplified RNA (aRNA) rather than on PCR. This technique produces an arithmetic amplification that might preserve proportional representation of individual cDNA species more faithfully than PCR. We also proposed that *in situ* first strand cDNA synthesis directly on a pathological section would improve first strand yield from paraffin embedded sections. We proposed to incorporate template switching into the first strand synthesis so that longer cDNA and longer aRNA would be obtained than from traditional methods. In last year's report, we delineated several problems we had encountered during the first year. The following summarizes those problems.

1. Simultaneous with a publication confirming our results[1], we discovered that the T_7 RNA polymerase is capable of considerable promoter-independent activity. These results imply that earlier work we had done with paraffin and frozen sections showing long aRNA were probably artifactual, resulting from promoter-independent activity of the T_7 RNA polymerase with co-purified DNA and RNA from the pathological section serving as the template. Consequently, if one wishes to use the aRNA method with T_7 RNA polymerase, mRNA or first strand cDNA must be purified following microdissection. Following double stranded cDNA synthesis, RNAse digestion should be done. In that way, the only high-affinity binding site for the T_7 RNA polymerase will be the promoter sequence appended to the double-stranded cDNA.

2. We showed that template switching events in solution were relatively rare. It is unlikely that template switching *in situ* will achieve the same frequency as in solution. Therefore, carefully designed experiments will be necessary to make sure that template switching is occurring at an acceptable frequency to achieve an accurate representation of gene expression. If results indicate that template switching is limiting our ability to produce good quality 2nd strand cDNA, this approach should be abandoned.

Because of these problems, the statement of work included with the original proposal is not applicable. We have been forced to step back and examine each step of the process to make sure we are using the most efficient methods with the highest yields. These steps are as follows:

1. mRNA and/or 1st strand purification.
2. First strand synthesis.
3. Second strand synthesis.
4. Amplification.

The following report delineates our progress thus far in examining these steps. We also identify additional issues and future experiments to lead to a successful conclusion to this project.

BODY:

1. Experiments to synthesize and purify 1st strand cDNA from small quantities of messenger RNA. Because of the promoter independent activity of the T7 RNA polymerase, the amplified RNA (aRNA) technique will be inefficient if contaminating RNA or DNA is included

in the aRNA synthesis reaction. For this reason, we have conducted a series of experiments to refine our techniques for synthesizing and purifying 1st strand cDNA from small amounts of mRNA.

1A. Purification of mRNA/1st strand cDNA with a biotinylated poly dT – T_3 or poly dT – T_7 primer. Because of the template-independent activity of the T_7 RNA polymerase and the need for purification of mRNA (above), we embarked on studies to capture either mRNA or 1st strand cDNA from a mixture of DNA and total RNA. To accomplish this we first used a biotinylated primer that consisted of 18 dT nucleotides with a T_3 or T_7 promoter sequence appended to the 5' end. Avidin magnetic beads were used to capture the biotinylated primer. We first tried this system on a mixture of DNA and total RNA in solution and then on a nucleotide mixture obtained from a frozen section.

1A.1. Purification of mRNA/1st strand cDNA from a DNA/total RNA mixture. A biotinylated $(dT)_{18}$ primer was synthesized with T_7 RNA polymerase promoter sequences appended to the 5' end (T_7–dT_{18}). To eliminate unbiotinylated primer, avidin beads (Roche) were incubated with a primer solution followed by thorough washing. The primer/bead complexes were then placed in a 20 µl RT reaction with 100 ng genomic DNA, 25 ng contaminating amplified RNA without poly A sequences, and 20 ng total RNA (which might be expected to contain approximately 1 ng mRNA). A similar reaction mixture was prepared that contained only 2 ng total RNA. The reaction mixtures were divided in half and one of the each halves was heated to 95° for 3 minutes to inactivate the RT enzyme. All reactions were then incubated at 42° for 1 hour. Following reverse transcription, beads were washed extensively with magnetic aggregation between washes and half of each RT reaction was subjected to PCR using GAPDH primers. At this point, because of dilutions at each step, products from approximately 1/10 of each original amount of total RNA were present. A positive control PCR reaction was included that contained a reaction with 2 pg total RNA but performed without beads, entirely in solution. In Figure 1, it is seen that the lane with the positive control contains a 430 bp GAPDH product, which is absent from the negative control in lane 2. In lanes containing the reactions in which beads were used, the same product is present in lanes from reactions in which in the RT was not heat-inactivated, but no product is present when the RT was heat-inactivated. These results show that contaminating DNA is not responsible for the observed band and therefore imply that the beads did not capture contaminating DNA.

1A.2. Purification of mRNA/1st strand cDNA from a frozen section. RNA was harvested from a 5 µm frozen section by scraping the tissue from the slide into 250 µl Triazol reagent (Life Technologies). The manufacturer's directions were followed for RNA extraction and precipitation. The pellet was suspended in 14.5 µl H_2O and stored at –80° until needed. To digest any co-purified DNA, DNAse I (1 units, Epicentre) was added in buffer supplied by the manufacturer along with 0.5 µl (20 units) RNAsin (Promega) and 2 µl 100 mM DTT in a total volume of 20 µl. The digestion was incubated at 37° for 1 hr. and then added, with 9.5 µl 20X SSC, to 2 µl avidin beads that had been pre-incubated with a biotinylated $(dT)_{20}$ primer with T7 RNA polymerase promoter sequences appended to the 5' end. After a 30-minute incubation at 37°, the beads were aggregated magnetically and the supernatant harvested to a new tube. Fresh beads, pre-incubated with the biotinylated primer, were added to this tube to capture any remaining mRNA. All beads were then incubated with the RT enzyme for 1 hr at 37° with

shaking to keep the beads suspended. Parallel reactions were included in which the RT enzyme was first inactivated by heating to 95° for 3 minutes. Following the RT reaction, the beads were washed extensively as above and reactions were diluted 1:10, 1:100, 1:1000, or 1:10,000 for PCR using GAPDH primers. A PCR reaction with positive control cDNA synthesized in solution was included. Figure 2 shows the results of this experiment. There is no GAPDH band present in the PCR reactions done on RT reactions in which the RT was inactivated, implying that the bands do not result from co-purified DNA. All dilutions of the RT reactions that contained active enzyme yielded appropriately sized bands. The dilutions of the RT reaction that came from the initial harvest of mRNA contain the strongest bands, while the dilutions of the RT reaction from the supernatant of the first reaction contain approximately 10-fold weaker bands. This experiment shows that mRNA can be harvested from a frozen section, isolated with a biotinylated poly-dT primer, and used to synthesize single-stranded cDNA. However, the primer/bead combination did not quantitatively remove the mRNA from the solution since about 1/10 was left in the supernatant and was detected by re-incubating the supernatant with additional beads. It is possible that a longer incubation or addition beads/primers would result in a more quantitative harvest of mRNA.

In section 1A.1, we showed that a purification scheme employing beads without DNAse was capable of isolating pure mRNA/1st strand cDNA. Therefore, DNAse treatment is probably not necessary in the scheme described in this section. However, we did not attempt to make aRNA from this cDNA, and it is unclear whether extremely small amounts of copurified DNA that might be present in the absence of DNAse treatment might serve as a promoter-independent template for T7 RNA polymerase. Future experiments will address this issue.

We have observed similar sensitivity in the detection of GAPDH cDNA from a frozen section in reactions carried out in solution without bead purification (not shown). However, our aim is not to detect one particular cDNA but to obtain a library of cDNAs that can be amplified by PCR or aRNA methods and subjected to analysis. Moreover, we will be harvesting only a few cells from a section, not the entire section as was done in these experiments. The purpose of the beads, therefore, is to quantitatively capture very small amounts of mRNA/1st strand cDNA so that it can be used to generate larger populations. As in section 1A.1, future experiments will address the quantitative aspects of this technique.

1B. Purification of mRNA/1st strand cDNA from a few cells. MCF-7 breast carcinoma cells were harvested, fixed with 70% ethanol and stained with eosin. An aliquot was counted in a hemocytometer and cells were diluted to a concentration of approximately 12 cells/µl. RNA was extracted from 1 µl of the suspension with the StrataPrep kit and resuspended in 30 µl. Approximately one-fourth of the RNA was used in each of 4 RT reactions. In 2 of these reactions, the RT enzyme was inactivated by heating to 95° for 3 minutes. One-fifth and 1/50th of each RT reaction were used as template for PCR reactions with GAPDH primers. RT reactions using 1 ng of total RNA as template served as positive controls. In Figure 3, it is seen that appropriately sized bands were obtained in all cases. These experiments show that RNA can be isolated from very few cells and that single strand cDNA can be synthesized from these cells.

1C. Improvement of 1st strand yield with an RNAse inhibitor. Some RT enzyme preparations, other reagents used in 1st strand synthesis, or components of single cells from

microdissection could contain small amounts of RNAses. This small RNAse activity might not be significant when large amounts of mRNA serve as template for an RT reaction. However, for the very small amounts of mRNA obtained from single microdissected cells, any small RNAse activity might produce a serious problem. We assessed the effect of including RNAse inhibitors in the RT reaction with 1 pg or 0.2 pg of total RNA serving as template and a $(dT)_{18}$ primer. In Figure 4, 1st strand cDNA from reactions with or without added RNAse inhibitors at concentrations recommended by their manufacturers were used as template for PCR reactions with GAPDH primers. The two RNAse inhibitors used in Figure 4 (RNAsin (Promega) and SUPERase-In (Ambion) both improved 1st strand yield because GAPDH bands are visible, whereas they are not in lanes where H_2O was added instead of an RNAse inhibitor. RNAsin had the most beneficial effect. In a separate experiment (not shown), gene 32 (Ammersham), a product that improves processivity of polymerases, was not able to increase yields over that achieved by RNAsin. These experiments demonstrate that inclusion of an RNAse inhibitor in the RT reaction mixture could be beneficial when dealing with small amounts of RNA template, as would be the case in microdissection.

2. Improving the yield in 2nd strand synthesis. One reason that it is important to maximize the yield of 1st strand cDNA synthesis (above) is that the synthesis of the 2nd strand is an inefficient process. In fact, in our hands, it is the most inefficient step that we have investigated. We had hoped that template switching would improve the efficiency of 2nd strand synthesis. However, for last year's report, we included data to show that template switching is a relatively rare event. Therefore, in this year, we have investigated several strategies to improve the efficiency of 2nd strand synthesis. We are designing strategies that can be used in either PCR amplification or in aRNA amplification. That way, we will have maximum flexibility in the final step of production of large amounts of cDNA from microdissected cells.

2A. Digestion of RNA. For maximum efficiency in 2nd strand synthesis, it is best if the RNA that served as template for the RT reaction is digested. This is important for 2 reasons. First, the second strand cannot be synthesized if the RNA/DNA duplex from the RT reaction is still intact because the primer and 2nd strand enzyme will not be able to bind to the 1st strand. Second, the RNA from the 1st strand reaction may compete with the 1st strand itself for binding to the 2nd strand polymerase. Although this competition would be relatively weak, as DNA polymerases bind preferentially to DNA, in working with such a little amount of template as we are, we must eliminate anything that would possibly interfere with the efficiency of the reaction. Therefore, we investigated ways to degrade the RNA while leaving the 1st strand cDNA intact. The starting material for digestion was a mixture of synthetic RNA and synthetic double-stranded DNA. We tested the RNAses in RT buffer (lanes marked H_2O, Figure 5) or in the buffer supplied by the manufacturer (lanes marked buffer). The negative control lane of Figure 5 shows the double stranded DNA as a bright band at about 400 bp. The RNA, which has a variety of lengths, is shown in the bright smear below the band of DNA. In subsequent lanes, the bright smear is less bright because the RNA has been digested. In the lanes for NaOH at 37° or RNAse One (Promega), the smear is not seen, implying that a large portion of the RNA has been digested so that it does not show up in the stained gel. In the lane for RNAse A, the smear does not show, but the double-stranded DNA has also been digested. The preparation of RNAse A we used possibly also contained DNAses. In a separate experiment, we showed that NaOH treatment did

not degrade single-stranded DNA and has no influence on subsequent PCR reactions (not shown).

2B. Use of a degenerate T_7 tagged primer and validation of the utility of the Klenow fragment for second strand synthesis. In last year's report, we showed that template switching utilizing terminal C's appended to the 1st strand cDNA by the SuperScript reverse transcriptase was a rare event. We wanted to try to improve the yield of second strand synthesis while still retaining a known sequence at the 5' end of the newly synthesized 2nd strand, as template switching does. We devised a 2nd strand primer consisting of a random septamer at the 3' end with the T_7 RNA polymerase promoter sequences at the 5' end (T_7–N_7). To test this primer in its ability to prime 2nd strand synthesis, we used purified 1st strand cDNA that was synthesized with a T_3–dT_{18} primer which had promoter sequences for the T_3 RNA polymerase appended to its 5' end. A 1:10 dilution of the purified single stranded cDNA was divided equally among twenty 2nd strand reactions and incubated with the T_7–N_7 primer and one of four DNA polymerases, each at multiple concentrations, to synthesize 2nd strand cDNA. It should be noted that the degenerate 2nd strand primer will hybridize to many places in the 1st strand, some at the far 3' end and some closer to the 5' end. Therefore, for each 1st strand cDNA species, we will get a family of 2nd strands, some long and some short. In fact, the same molecule of 1st strand cDNA can serve as template for multiple 2nd strand primers and synthetic reactions simultaneously.

Because the poly-dT primer for 1st strand synthesis was tagged with the T_3 RNA polymerase promoter sequences and the T_7–N_7 primer for 2nd strand synthesis was tagged with the T_7 RNA polymerase promoter sequences, we could use commercial T_3 and T_7 primers in PCR to amplify the cDNA population. Therefore, following 2nd strand synthesis, 1/10th of the product of each 2nd strand reaction was used for a PCR reaction primed by the T_3 and T_7 primers. One-thousandth of this first PCR reaction was used for a subsequent PCR reaction with GAPDH primers. Figure 6 shows a scheme of the process and the results of the PCR reactions for GAPDH. All DNA polymerases tested except SuperScript II (Life Technologies) were able to complete 2nd strand synthesis. However, the Klenow fragment (Promega) was able to do so most robustly. A puzzling feature of the reactions with Sequenase (USB) and Improm II (Promega) is the presence of a shorter product in reactions using higher concentrations of these enzymes. As the concentration of enzyme decreases, an appropriately sized product becomes visible. In the lane for Sequenase used at 13 units, both products are present. Since the products would not have been amplified if the T_3 and T_7 sequences and the GAPDH primer sites were not present, it would seem that the shorter product is the result of some sort of internal splicing. In any case, we have determined that the Klenow fragment is the most dependable enzyme for 2nd strand synthesis.

3. Representational amplification of cDNA. Because the goal of the project is to obtain representative amplified populations of cDNA from microdissected cells, we studied two methods to amplify entire cDNA populations produced by methods derived from the above experiments. First, we can use PCR with primers to those tags to amplify the entire population. Alternatively, we can use the T7 RNA polymerase promoter tag on the 5' end of the cDNA population to generate amplified RNA. We are investigating both these techniques to see which proves to be the most useful.

3A. Amplification of cDNA populations with PCR. We synthesized cDNA from a mouse fibroblast cell line to demonstrate the utility of PCR for cDNA population amplification because we have on hand many primer sets for cDNAs that should be present in the population from that cell line. We used these specific primers to monitor the progress of the amplification at every step. Total RNA from Swiss 3T3 cells was used to synthesize 1st strand cDNA, which was treated with alkali, RNAse One? and purified. An aliquot was saved to be amplified with the specific primers. The 1st strand cDNA was diluted 1:100 and used in a PCR reaction with T_7 and T_3 primers under the following conditions: 94° for 2 minutes followed by 35 cycles of 94° for 20 seconds, 55° for 30 seconds and 72° for 1 minute. Aliquots of the product were taken for amplification with the specific primers and a 1:1000 dilution of the product was used for a 2nd round PCR reaction. The 2nd round PCR product was also used for amplification with specific primers. The results of this experiment are shown in Figure 7. Two rounds of PCR amplification resulted in an approximately million-fold amplification of the specific cDNAs with apparent preservation of their relative abundance as shown by this admittedly approximate method of quantitation. Separate experiments using longer extension times and long-distance polymerase mixtures did not improve the results (not shown).

The above results were very encouraging, and we felt they could be improved upon with optimization of the PCR conditions for the population amplification. In particular, we felt that fewer cycles for each round of PCR might preserve fidelity of representation more faithfully since it would be more likely that each transcript would be amplified in a strictly logarithmic fashion if cycle number were limited somewhat. If that were the case, we could possibly carry out more rounds of PCR amplification, thereby obtaining more cDNA. We therefore carried out the process depicted in Figure 7 to 5 rounds of PCR amplification with T_7 and T_3 primers, testing the transcripts of individual cDNAs in each round. With 35 cycles of PCR in each round, individual genes were poorly represented in the 5th round cDNA population (not shown). However, by shortening each PCR round to 20 cycles, we obtained good representation of each individual transcript in the 5th round cDNA population (Figure 8A, 8B, and 8C).

These experiments show that PCR can be used to amplify a cDNA population. However, PCR testing of individual transcripts is a rough way to estimate fidelity of representation in an amplified population. During the next year, we will be testing these PCR methods with cDNA microarrays (see "Conclusions").

3B. Amplification of cDNA populations with amplified RNA. In a "proof of principle" test of the method, PCR amplification product from 2nd and 3rd round amplification as depicted in Figure 7 was used to produce aRNA. The double-stranded cDNA was first purified with the Qiagen PCR purification kit and then divided between two reaction mixtures with appropriate components but with or without T_7 RNA polymerase. After a 2-hour incubation at 37°, the reaction mixtures were treated with DNAse I for 20 minutes at 37°. RNA was extracted with Triazol and precipitated. Pellets were resuspended in 20 µl H_2O and 5 µl of that was subjected to reverse transcription in RT reactions using the T_3–dT_{18} primer. One of these reactions contained heat-inactivated RT enzyme. Negative control reactions included one with template from the aRNA reaction in which the T_7 RNA polymerase was omitted and one with no RT. A positive control reaction containing 10 pg total RNA from Swiss 3T3 cells was also done. Following the

RT reaction, PCR with GAPDH primers confirmed the presence of 1st strand cDNA (Figure 9, lane 7). A lane with total aRNA shows a bright smear, while an RT-PCR reaction on a diluted aliquot of the aRNA produced a GAPDH band. Additionally, no GAPDH band and no smear were present in the negative controls. The cDNA synthesized from the aRNA was used in PCR reactions for individual cDNA species, as was done in the PCR amplification. These reactions showed that most of the individual species were present (Figure 8D).

These experiments are in their first stages and the method needs additional refinement. However, we are encouraged that our method has yielded aRNA and will continue to optimize conditions with the goal of ultimately being able to compare the aRNA method with the PCR method on cDNA microarrays. Moreover, it is possible that we may be able to combine the two methods to give more faithful representation of individual cDNAs in amplified populations.

4. Repeated aRNA production after cRNA capture on magnetic beads. We showed above that we were able to capture mRNA/1st strand cDNA on magnetic beads. We wondered whether double-stranded cDNA captured in the same way could produced repeated batches of aRNA. We synthesized a test template cDNA by using a biotinylated T_7–dT_{18} primer to synthesize first strand cDNA from total RNA and then used a sense GAPDH primer with the same poly–dT primer in 3' RACE PCR to synthesize a double-stranded GAPDH cDNA. This product was purified with the Qiagen PCR purification kit and captured with avidin magnetic beads. Aliquots of the beads with attached GAPDH cDNA were incubated in a 15 µl T_7 RNA polymerase reaction with shaking for 2 hours, followed by harvest of the supernatant and washing of the beads. The same reaction was repeated 7 times with fresh reagents, with washing of beads between each reaction. Supernatants were extracted with Triazol, precipitated and resuspended in 20 µl of H_2O. Aliquots of each reaction were electrophoresed on a non-denaturing gel. The product of the first reaction was diluted to 80%, 60% and 40% for comparison of subsequent reactions. Figure 10 shows the aRNA products of each of these reactions. It can be seen that the yield of each reaction is slightly less than the preceding one, but that all reactions contain the aRNA product (the smears above and below the expected product are encountered because of the use of the non-denaturing gel, which is used because of its superior sensitivity in ethidium bromide staining). This experiments show that repeated aRNA synthesis reaction from a cDNA template immobilized on magnetic beads is possible and would at least theoretically result in increased yields of aRNA.

KEY RESEARCH OUTCOMES:

- mRNA/1st strand cDNA can be purified from RNA/DNA mixtures, including those obtained from frozen sections or from just a few cells, by use of a biotinylated poly–dT primer and avidin magnetic beads.
- Use of RNAsin, an RNAse inhibitor, improves yields of 1st strand cDNA.
- A combination of alkali and RNAse One can be used to efficiently digest RNA after 1st strand cDNA synthesis without degrading the single-stranded cDNA.

- A degenerate T_7–N_7 primer can be used to prime 2nd strand cDNA synthesis and append the T_7 RNA polymerase promoter sequences to the 5' end of the 2nd strand cDNA.

- PCR amplification of a total cDNA population using appended T_3 and T_7 tags produces representational cDNA after 5 rounds of PCR.

- Repeated rounds of aRNA synthesis from the same cDNA template immobilized on magnetic beads produces additional aRNA.

REPORTABLE OUTCOMES:

McLeskey, S.W., Eberwine, J.H., and Huang, C. Amplification of small quantities of messenger RNA. Era of Hope, Department of Defense Breast Cancer Meeting, Orlando, FL, September 25-28, 2002.

CONCLUSIONS: We have developed efficient ways of synthesizing 1st strand cDNA that include isolation with magnetic beads and of improving the yield of 2nd strand cDNA. The use of a degenerate, tagged primer for 2nd strand synthesis gives us a choice of two ways of amplifying the cDNA — PCR or amplified RNA. Future experiments to optimize each step of the process will be culminated by validation of their fidelity with the use of microarrays. Remaining questions for each step of the process are delineated below:

1. **mRNA and/or 1st strand purification and 1st strand synthesis.** Further experiments with magnetic beads are needed to delineate the optimal time of incubation and quantity of beads necessary to remove a higher percentage of mRNA/1st strand cDNA from solution. We estimate that we got approximately 90% harvest with the bead quantity and incubation time described in section 1A. However, it is possible this could be improved. We will also confirm our preliminary results that DNAse treatment is not necessary when bead purification is used, even if the cDNA will be used for aRNA synthesis. Finally, we will use the bead purification techniques on microdissected material rather than the whole slide to confirm their utility in cases where the amount of mRNA/1st strand cDNA is very small. With respect to 1st strand synthesis, further experiments with RNAse inhibitors, incubation times, and enzyme concentrations for microdissected samples will optimize this process.

2. **2nd strand synthesis.** Future experiments will determine optimal concentrations of the N_7–T_7 primer and the Klenow enzyme. These will be done on experimentally generated 1st strand cDNA. The results from these experiments will then be applied to 1st strand cDNA generated from microdissected cells from pathological sections.

3. **Amplification of cDNA.** Further work needs to be done to optimize the PCR and aRNA amplification using 1st strand cDNA generated experimentally. In this way, quantitation of yields can be done to improve yields at every step. Our rough estimation of fidelity using the panel of specific primers can be used to make sure that at least those transcripts are preserved

throughout the amplification process. When we have gone as far as we can with these methods, we will use cDNA microarrays to test our amplifications. By using experimentally generated cDNAs, we will be able to compare cDNA made with traditional techniques from the source RNA with the amplified cDNA populations originating from small amounts of source RNA. We will compare various rounds of PCR and aRNA amplification with each other and with cDNA made directly from larger amounts of the source RNA.

4. *In situ* 1st strand synthesis on paraffin and frozen sections. The main thrust of the project as it was originally proposed was to be able to employ the technique of *in situ* first strand synthesis on paraffin sections to see if that resulted in useable cDNA populations. These experiments were put on hold when we discovered problems with later steps in the process, as explained in "Introduction". However, in the final year we will be conducting experiments to see if this approach is viable and whether it results in improved cDNA populations when compared with microdissecting the cells and synthesizing the 1st strand in solution.

This project has been extremely challenging but we have made significant progress in the past year. It is likely that we will be finalizing our approach in coming months, and submitting a manuscript for publication.

REFERENCE LIST:

1. Baugh, L. R., Hill, A. A., Brown, E. L., and Hunter, C. P. Quantitative analysis of mRNA amplification by in vitro transcription. Nucleic Acids Res., 29: E29. 3-1-2001.

APPENDIX

1. List of acronyms.

2. Abstract presented at the 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, CA, April 6-10, 2002.

cDNA	Copy DNA
DTT	Dithiothreitol
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
PCR	Polymerase chain reaction
RNA	Ribosenucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase – polymerase chain reaction
SSC	sodium chloride/sodium citrate solution
UMB	University of Maryland at Baltimore

Abstract presented at the 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, CA, April 6-10, 2002

Differential Gene Expression in Tumor-Associated Endothelium

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Although tumor-associated blood vessels have unique morphology and function, little is known about expression of genes that might determine their phenotype. We have performed a one-step flow cytometric separation of endothelial cells from MCF-7 xenograft tumors growing in nude mice or from mouse mammary fat pad. RNA was immediately extracted from the sorted cells and subjected to amplified fragment length polymorphism analysis. To date, we have about 30 candidate genes that may be differentially expressed and are confirming that expression in our xenografts and in human breast cancer. These genes include members of the tissue plasminogen activator/inhibitor family, proteins involved in cell-to-cell communication and motility, and unknown ESTs. Our current results concerning differentially expressed genes will be presented.

APPENDIX

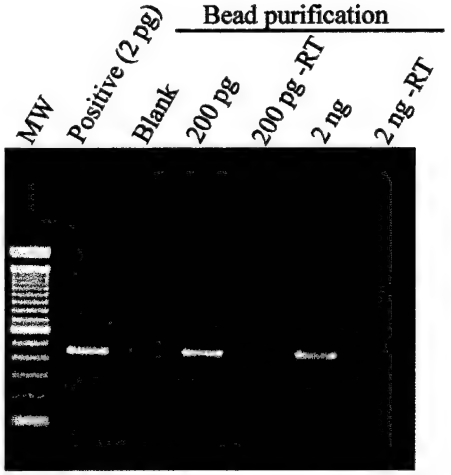


Figure 1. Bead purification of mRNA from small amounts of total RNA does not contain contaminating DNA. mRNA purified from a mixture of total RNA and genomic DNA with magnetic avidin beads preincubated with a biotinylated poly-dT primer was used for RT reactions. The resultant single-stranded cDNA was used in PCR reactions with GAPDH primers. -RT, the RT enzyme was heat-inactivated. The quantity of total RNA used as starting material is indicated above each lane.

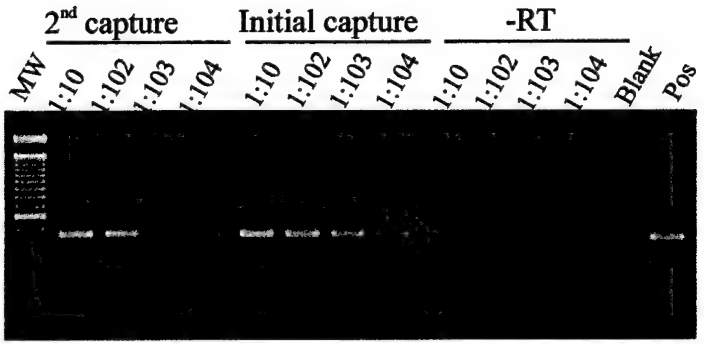


Figure 2. 1st strand synthesis from captured mRNA from a frozen section. Total RNA was extracted from a frozen section and used for template in an RT reaction in which the biotinylated poly-dT primer had been captured with avidin beads. After the initial capture of mRNA by the immobilized primer, the beads were magnetically aggregated and supernatant was harvested and incubated with additional primer-bead combinations to detect residual mRNA remaining after the 1st capture. RT reactions were carried out on the first capture, the second capture, and an additional initial capture in which the RT enzyme was heat-inactivated. Single stranded cDNA was harvested, diluted as indicated, and used for PCR reactions with specific GAPDH primer. The positive control was a similar RT-PCR done completely in solution.

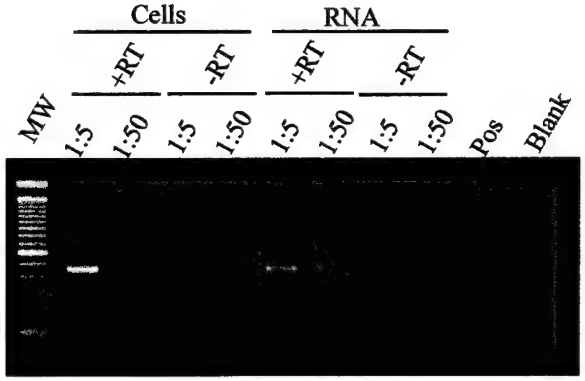


Figure 3. Purification of mRNA/1st strand cDNA from a few cells. RNA from approximately 12 breast carcinoma cells was column purified and used as template for RT reactions that included active enzyme or heat-inactivated enzyme (-RT). One-fifth or 1/50th of each RT reaction was used as template for a PCR reaction with GAPDH primers. 1 ng total RNA from the same cells was used in similar reactions for comparison. The expected 430 bp GAPDH product is seen in all lanes except those in which the RT was heat-inactivated.

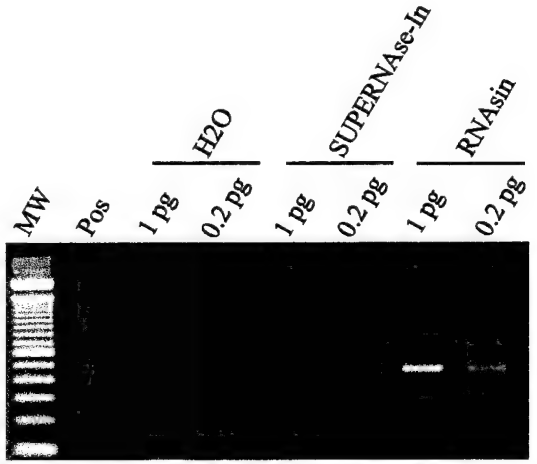


Figure 4. Effect of including RNAse inhibitors in the RT reaction. 1st strand cDNA synthesized in RT reactions in which the indicated RNAse inhibitors were included was used for PCR reactions with GAPDH primers. The 1st strand reactions were done in RT buffer (H_2O lanes) or in RT buffer plus the indicated RNAse inhibitors.

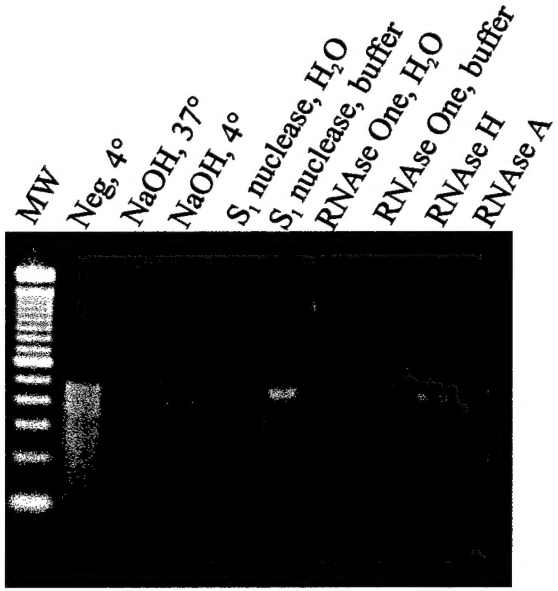


Figure 5. Digestion of RNA with various treatments. In the neg, 4° lane, double stranded DNA (the band at approximately 475 bp and synthetic RNA (the bright smear under the DNA band) are undigested. In subsequent lanes, the indicated treatments were added to assess their ability to digest RNA while leaving DNA intact. The RNAses were added to RT buffer (H_20 lanes), or to reactions with their recommended buffer (buffer lanes). Of the RNAses tested, RNAse One and alkali provided the most complete digestion of RNA without degrading DNA..

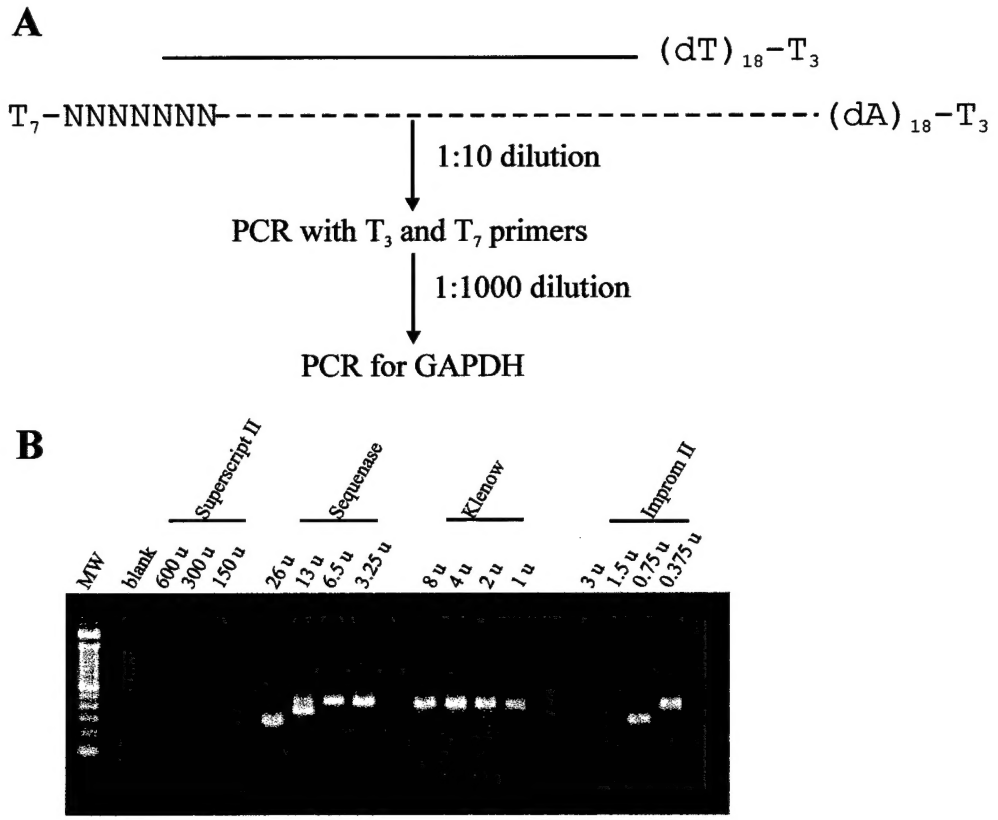
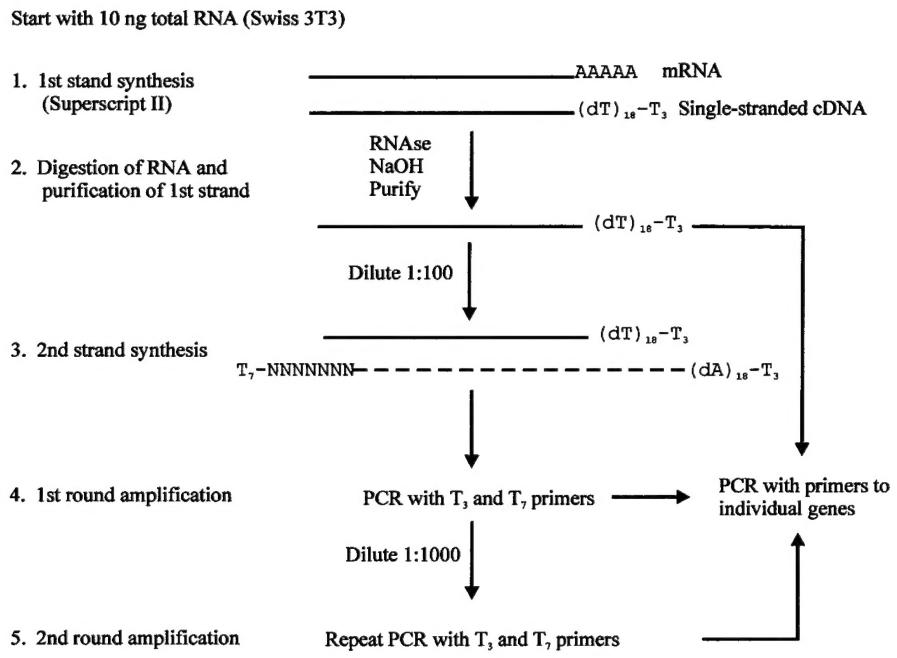
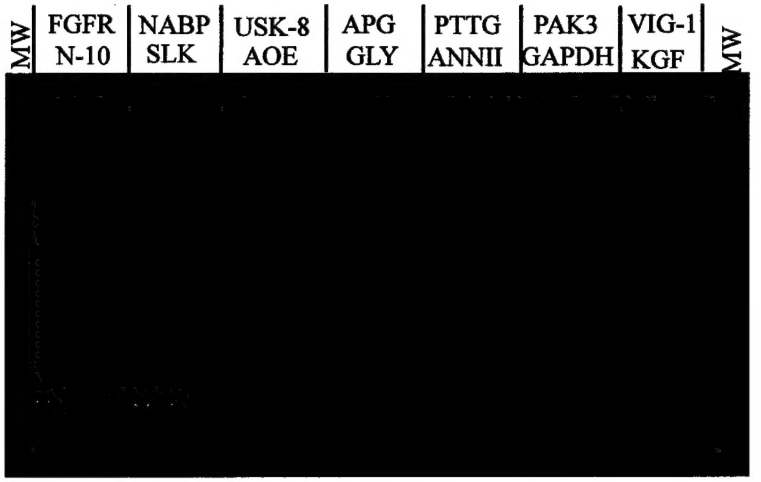


Figure 6. The Klenow fragment is a good enzyme for 2nd strand synthesis with a degenerate tagge primer. Equal amounts of single-stranded cDNA were incubated in 2nd strand reactions using the T_7-N_7 prime Enzymes and amounts used are as indicated. Buffers were as stipulated by the manufacturer. Following 2r strand synthesis, one-tenth of the total cDNA population was amplified with T_3 and T_7 primers and on thousandth of that reaction was used with specific primers for GAPDH (429 bp product).

A. Experimental Scheme for Amplification of cDNA



B



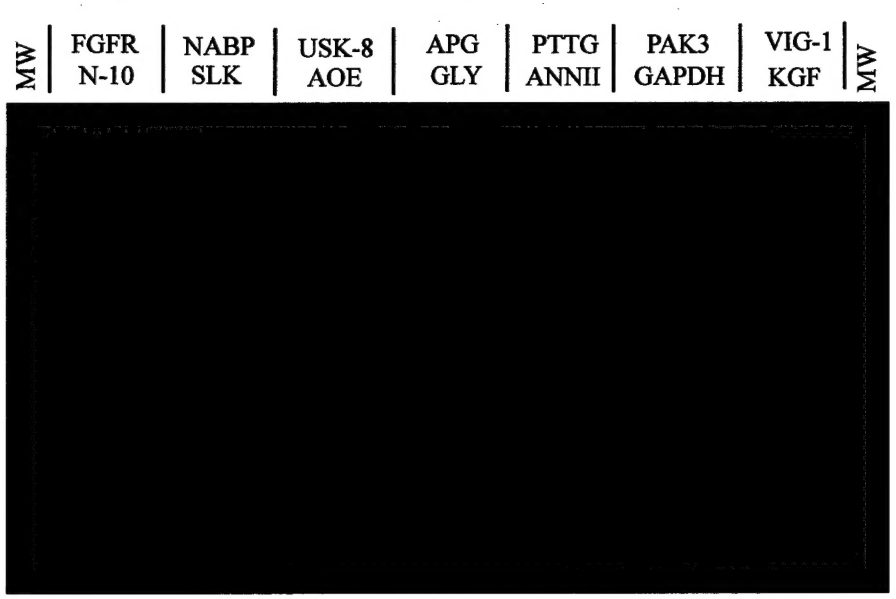
Single-stranded

1st round PCR

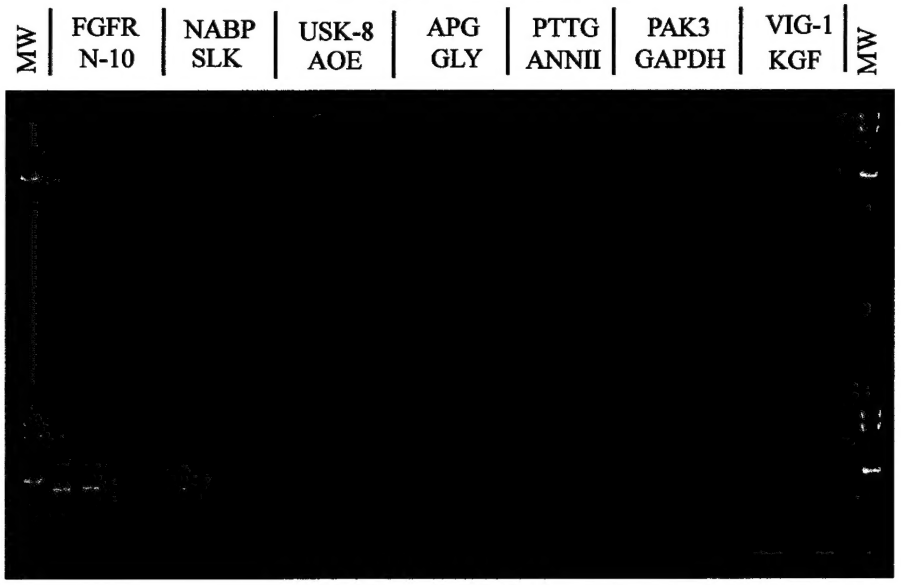
2nd round PCR

Figure 7. A. Scheme for producing multiple rounds of amplified cDNA. 1st and 2nd strand cDNA were produced according to protocols developed previously, then diluted and subjected to PCR with T_3 and T_7 primers. The PCR reactions were diluted and amplified again to produce 2nd round amplified cDNA. Aliquots at each step were reserved for amplification of specific transcripts as indicated. **B. Comparison of individual transcript abundance for selected genes after 2 rounds of PCR amplification.** After amplification of the entire cDNA population (see scheme), the abundance of representative genes was tested using primers specific for particular cDNAs as indicated. Each cDNA's representation in the population is approximately 1000-fold increased in the 1st round PCR and approximately one million-fold increased in the 2nd round PCR. However, a few genes, such as FGFR and GLY, are not amplified at the same rate as the rest.

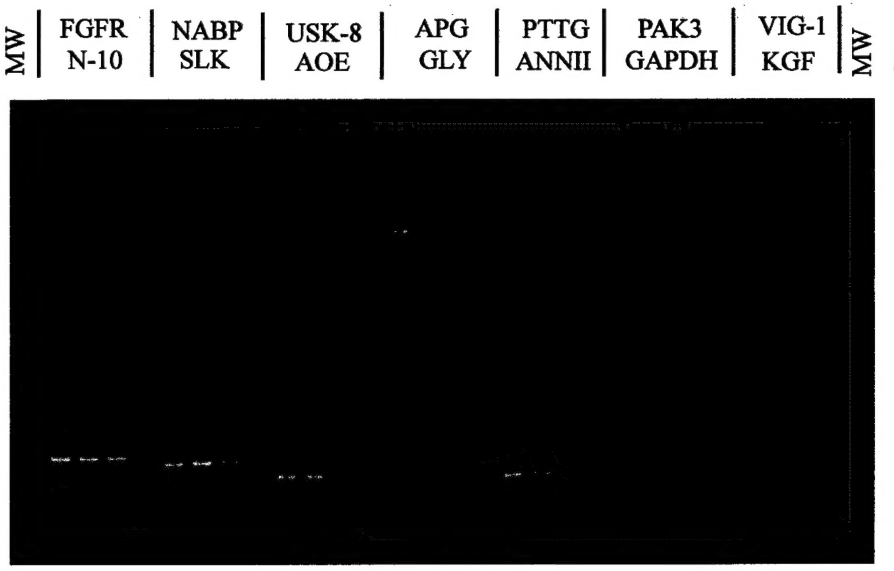
For each gene, each successive lane shows PCR product for a 10-fold dilution of template from the previous lane. The most concentrated template for single-stranded cDNA was 200 pg/µl. Reactions for 1st and 2nd round PCR were diluted in the same way as for the single-stranded cDNA.



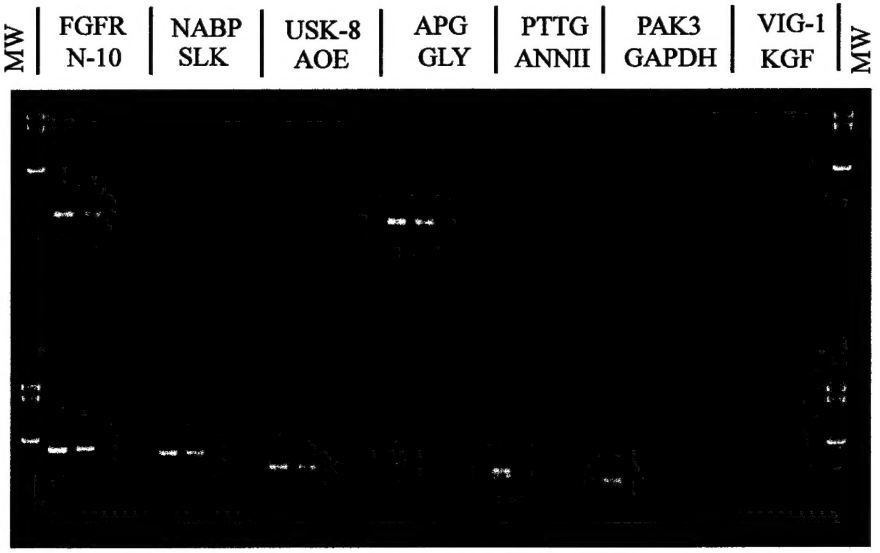
A. Single-stranded cDNA



B. 1st round cDNA



C. 5th round cDNA



D. Amplified RNA

Figure 8. Amplification of cDNA populations by PCR or aRNA. A-C. Purified single stranded cDNA was amplified in PCR reactions with T_3 and T_7 primers in the same manner as for Figure 7 except that the PCR reactions for each round were done for 20 cycles instead of 35. Aliquots of the original single-stranded cDNA and PCR products from the 1st and 5th rounds of PCR amplification were subjected to PCR analysis with primers for specific transcripts, as shown. **D.** An aliquot of double-stranded cDNA from 2nd and 3rd round PCR amplification was used as template for second strand synthesis and aRNA production. The aRNA was used as template for RT-PCR reactions with the transcript-specific primers.

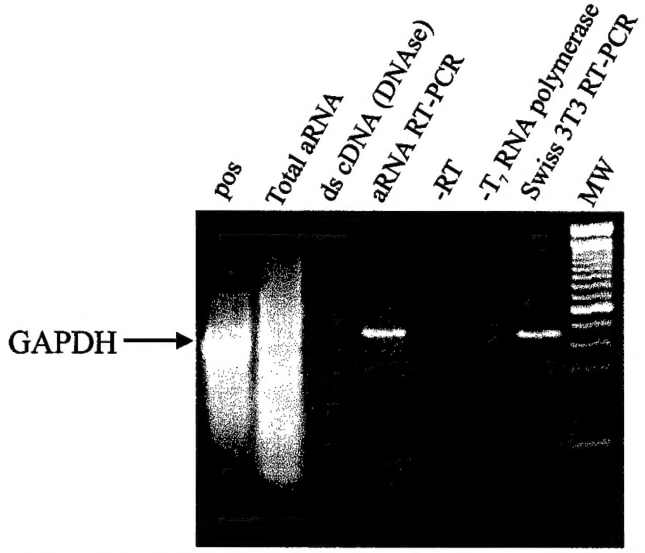


Figure 9. RT-PCR with primers for GAPDH shows the presence of aRNA populations. Double-stranded cDNA was synthesized from Swiss 3T3 cell RNA with the T_7 RNA polymerase promoter appended to the 5' end. Following DNAse treatment (lane 3), aRNA was produced (lane 2). aRNA was subjected to an RT-PCR reaction with specific primers for GAPDH (lane 4). In lane 7, 10 ng total RNA from Swiss 3T3 cells was used as template for GAPDH RT-PCR. Lane 5 contains products of an aRNA reaction with active T_7 but no RT, and lane 6 contains products with no T_7 but active RT. Lane 1 is a positive control GAPDH RNA produced by direct transcription.

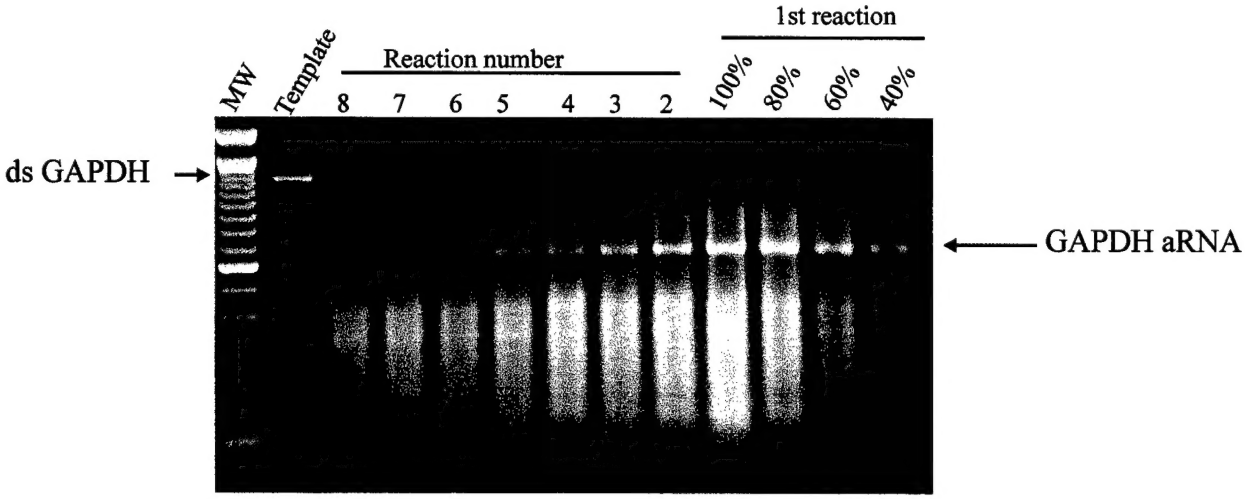


Figure 10. Amplified RNA produced by repeated rounds of aRNA synthesis from immobilized double-stranded cDNA. Double stranded cDNA was synthesized by 3' RACE PCR with a biotinylated T_7-dT_{18} primer linked to avidin magnetic beads and a sense GAPDH primer. This cDNA was used to repeatedly synthesize aRNA in 8 successive reactions in which T_7 RNA polymerase and appropriate reaction components were incubated with the beads and the supernatant harvested. The double-stranded cDNA and expected aRNA product are indicated by arrows. Numerals indicate the number of the reaction. The first reaction has been diluted at the concentrations indicated.